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Review

Cytochrome b_{559} and cyclic electron transfer within photosystem II

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ABSTRACT

Cytochrome b_{559} (Cyt b_{559}), β -carotene (Car), and chlorophyll (Chl) cofactors participate in the secondary electron-transfer pathways in photosystem II (PSII), which are believed to protect PSII from photodamage under conditions in which the primary electron-donation pathway leading to water oxidation is inhibited. Among these cofactors, Cyt b_{559} is preferentially photooxidized under conditions in which the primary electron-donation pathway is blocked. When Cyt b_{559} is preoxidized, the photooxidation of several of the 11 Car and 35 Chl molecules present per PSII is observed. In this review, the discovery of the secondary electron donors, their structures and electron-transfer properties, and progress in the characterization of the secondary electron-transfer pathways are discussed. This article is part of a Special Issue entitled: Photosystem II.

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1. Introduction

Photosystem II (PSII) catalyzes the first step of oxygenic photosynthesis in which electrons are extracted from water, ultimately resulting in conversion of energy from the sun into chemical energy. Each time the energy from a photon of light reaches the reaction center of PSII, a charge separation occurs from a group of four chlorophylls denoted as P_A , P_B , B_A and B_B (Fig. 1); the primary electron donor is called P_{680} . The electron is transferred to a membrane-bound quinone, Q_A , via a pheophytin (Pheo_A) cofactor. The charge separation between P_{680} and Q_A , 26 Å apart, is long-lived with a time constant for recombination of at least 0.1 ms in higher plants [1–4] and 1 ms in cyanobacteria [2,5–9]. During electron transfer that results in water-oxidation catalysis, the hole remaining on P_{680} is transferred to the catalytic tetra-manganese cluster known as the oxygen-evolving complex (OEC) via a redox-active tyrosine, Y_Z , and the electron on Q_A is transferred to the terminal


electron acceptor in PSII, Q_B (reviewed in [10]). The OEC stores the oxidizing potential, cycling in single electron-transfer steps from S_0 (the least oxidized state) to S_4 (the most oxidized state), when it is capable of splitting water into O_2 , electrons, and protons. However, when P_{680}^{*+} , with a reduction potential of about 1.25 V [11,12], cannot oxidize the primary electron donors of PSII, which include Y_Z and the OEC, photoinhibition resulting in loss of activity can occur (reviewed in [13]). Potential sources of photoinhibition include oxidative damage to proteins and cofactors, or recombination of the $P_{680}^{*+}Q_A^{-\bullet}$ radical pair, forming $^3P_{680}$ that can sensitize the formation of singlet oxygen and other reactive oxygen species [14–18].

Under conditions in which electron transfer from the OEC is impaired, photooxidation of the redox-active tyrosine in the D2 subunit (Y_D), β -carotene (Car), chlorophyll (Chl), and cytochrome (Cyt) b_{559} has been observed (Fig. 1). These secondary pathways of electron transfer have been proposed to protect PSII from oxidative photodamage under high light conditions or while the OEC is undergoing assembly [19]. An oxidizing equivalent on Y_D does not equilibrate among the other secondary electron donors and, as such, Y_D serves as a singular secondary electron donor and typically remains oxidized under steady-state illumination of PSII. On the other hand, an oxidizing equivalent rapidly equilibrates among Cyt b_{559} , Car, and Chl [20]. As the lowest potential donor, Cyt b_{559} is preferentially oxidized when an oxidizing equivalent from P_{680}^{*+} equilibrates among these secondary electron donors. Oxidized Cyt b_{559} is then capable of accepting an electron from a reduced plastoquinone (PQ) on the electron acceptor side of PSII, thus forming a cyclic pathway of electron transfer that connects the donor and acceptor sides of PSII to remove excess oxidizing equivalents (see Section 2).

The one copy of Cyt b_{559} per PSII is located near the D2 subunit (Fig. 1A), slightly separated from the primary electron-transfer

Abbreviations: ADRY, acceleration of the deactivation reactions of the water-splitting system Y; Car, β -carotene; Car $^{*+}$, oxidized Car; CD, circular dichroism; Chl, chlorophyll; Chl $^{*+}$, oxidized Chl; Chl_{D1}/Chl_{D2}, redox-active Chl in the D1/D2 polypeptides; CP43/CP47, 43/47 kDa chlorophyll binding proteins in PSII; Cyt, cytochrome; D1/D2, homologous PSII reaction center core proteins; ENDOR, electron nuclear double resonance; EPR, electron paramagnetic resonance; ESEEM, electron spin echo envelope modulation; FTIR, Fourier transform infrared; HP, high potential; HPLC, high performance liquid chromatography; HYSCORE, hyperfine sub-level correlation; IP, intermediate potential; LD, linear dichroism; LP, low potential; Near-IR, near-infrared; OEC, oxygen-evolving complex; P_{680} , primary Chl electron donor in PSII; Pheo, pheophytin; PQ, plastoquinone; PSII, photosystem II; Q_A/Q_B , redox-active quinones bound to PSII; $S_0/S_1/S_2/S_3/S_4$, the redox states of the OEC; WT, wild type; Y_Z/Y_D , redox active tyrosines in the D1/D2 polypeptides

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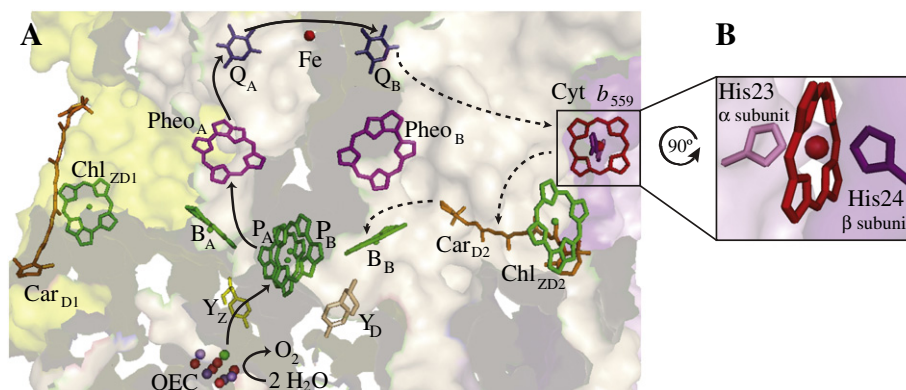


Fig. 1. A. Electron-transfer cofactors in photosystem II, viewed from along the membrane plane (PDB ID: 3ARC), with solid arrows showing the primary electron-transfer steps and dashed arrows showing secondary electron-transfer pathways. The oxygen-evolving complex (OEC) is shown with manganese atoms in purple, oxygen in red, and calcium in green; tyrosine Z (Y_Z) is shown in yellow and tyrosine D (Y_D) is shown in tan; chlorophylls (Chl) are shown in green; β -carotene (Car) is shown in orange; pheophytins (Pheo_A and Pheo_B) are shown in magenta; and quinones (Q_A and Q_B) are shown in blue. The surfaces of the D1, D2, α - and β -Cyt b_{559} subunits are colored yellow, tan, pink and purple, respectively. B. An enlarged view of Cyt b_{559} .

cofactors. The heme is ligated via two His residues, one each from the α and β Cyt b_{559} subunits, thereby forming a cross-linked structure (Fig. 1B). Cyt b_{559} displays a range of midpoint potentials depending on the type of PSII preparation and treatment: -150 – 0 mV (low potential or LP), $+230$ mV (intermediate potential or IP), and $+390$ mV (high potential or HP) (see Section 3). The structural differences among the different forms of Cyt b_{559} are unknown, and while many treatments have been observed to shift Cyt b_{559} from the HP to LP form *in vitro*, the restoration of the HP form is more difficult to achieve experimentally. However, it has been hypothesized that the interconversion may play a physiological role in mediating the operational type of photoprotection (see Section 2.1).

The connectivity and sequence of electron-transfer events among Cyt b_{559} and the multiple redox-active Car and Chl are of interest in understanding the characteristics of secondary electron transfer and its physiological relevance. Crystallographic studies have yielded detailed information on the structure of PSII and the locations of the cofactors (see Section 4); however, the redox-active cofactors must be identified by other spectroscopic methods (see Section 2). Recent studies have characterized secondary electron transfer under a variety of conditions, including in systems containing different redox forms of Cyt b_{559} and in both catalytically active (O_2 evolving) and inactive PSII samples (see Section 5). Other reviews provide additional information on the secondary donors [21–24] and Cyt b_{559} [19,25–27].

2. Spectroscopy identifying the cofactors involved in secondary electron transfer

2.1. The discovery of the redox activity of Cyt b_{559}

Although photosynthesis has been studied for more than a century, the redox-activity of the secondary cofactors, Car, Chl, and Cyt b_{559} , was discovered more recently. Photooxidation of the preferred electron donor in the secondary electron-transfer pathway, Cyt b_{559} , was observed by absorption spectroscopy at cryogenic temperatures [28,29]. An inverse relationship between Cyt b_{559} oxidation and S_2 state formation was observed by EPR spectroscopy, indicating that the two species are competing electron donors to P_{680}^{*+} [30]. Photooxidized Cyt b_{559} was also found to be photoreduced [31], and it was hypothesized that PQ was the source of electrons [26], creating a cyclic pathway around PSII hypothesized to decrease damage due to high light [32,33]. The presence of a cyclic pathway was confirmed at saturating light intensities by the greater yield of variable fluorescence, indicative of charge separation, than the yield of O_2 [34]. It was observed that the magnitude of photooxidation and photoreduction of Cyt b_{559} increased

under stress conditions, such as high temperature, implying a biological role for the cyclic pathway in photoprotection [35].

At room temperature, the rate of photooxidation of Cyt b_{559} in O_2 -evolving PSII samples ($t_{1/2} = 760 \pm 260$ ms at pH 6.0) is on the same order of magnitude as the rate of S_2 Q_A $^{*-}$ charge recombination [36], and the rate of Cyt b_{559} photooxidation in PSII samples inactive for O_2 -evolution ($t_{1/2} = 13$ ms at pH 6.0) is similar to the rate of Y_Z^{*+} Q_A $^{*-}$ charge recombination [7]. However, a higher yield of oxidized Cyt b_{559} was detected in non- O_2 -evolving PSII samples [7,35,36]. These observations were explained by a model where Cyt b_{559} is photooxidized via electron transfer from the reduced heme to P_{680}^{*+} , which is present in equilibrium with either the S_2 state or Y_Z^{*+} [7,36].

Following its photooxidation, Cyt b_{559} photoreduction has also been observed at pH ≥ 6 . In a sample of PSII-enriched membranes (BBY-type preparation), the $t_{1/2}$ is about 50 s [36], while in chloroplasts the $t_{1/2} = 100$ ms [26]. There have been several hypotheses regarding the source of the electrons, including the PQ pool [26], Q_B $^-$ [36], or Pheo $^-$ [37–39]. Based on the 1.9 Å resolution crystal structure [40], electron transfer to Cyt b_{559} from the Q_B site, 25 Å away, may not be possible with a $t_{1/2}$ of 100 ms [21], although it would be possible with a $t_{1/2}$ of 50 s. Electron transfer from Pheo $^-$ to Cyt b_{559} , at a distance of 38 Å, would not be possible with either $t_{1/2}$. There are significant differences between samples composed of chloroplasts and of PSII-enriched membranes, including the size of the PQ pool, the condition of the Q_B site, and the presence or absence of gradients across the chloroplast membrane; also, the source of electrons may be different in the two samples.

Under some conditions, photoinhibition is decreased when LP Cyt b_{559} is preoxidized and available for reduction by the acceptor side of PSII [37–39,41]. However, increasing the amount of LP Cyt b_{559} has not yielded an appreciable decrease in photoinhibition at high light intensities [42]. Still, it has been hypothesized that HP and LP Cyt b_{559} may interconvert reversibly [41], with HP Cyt b_{559} , which is reduced under ambient conditions, poised to donate an electron to P_{680}^{*+} under donor-side stress, and LP Cyt b_{559} , which is predominantly oxidized under ambient conditions, poised to accept an electron under acceptor-side stress in which excess electrons accumulate on Q_A and Pheo [37–39,41,43].

2.2. Observations of Chl $^{*+}$ and Car $^{*+}$ before 2000

The photobleaching of neutral Car [44] and Chl [45] was first observed in chloroplasts with illumination under oxidizing conditions. Later, flash-induced absorbance changes showed Car bleaching concomitant with Cyt b_{559} reduction at room temperature in the presence of an ADRY reagent, which disrupts normal electron transfer [46]. Car $^{*+}$ was also observed by flash absorption spectroscopy at room

temperature in the presence of phenolic herbicides with a $t_{1/2} = 10\text{--}35\ \mu\text{s}$ [47]. At cryogenic temperature, it was shown that Car bleaching occurred without ADY reagents or herbicides, and was due to $\text{Car}^{\bullet+}$ formation, marked by an absorbance peak at 990 nm (see Fig. 3) in conjunction with bleaching at 480 nm [48]. Samples with preoxidized Cyt b_{559} yielded a greater absorbance at 990 nm, providing a clue that Car and Cyt b_{559} are members of the same electron-transfer pathway [48]. This was reinforced by the detection of an EPR signal attributed to $\text{Chl}^{\bullet+}$ or $\text{Car}^{\bullet+}$ present only in samples with Cyt b_{559} preoxidized [30,49]. Additionally, Chl (or Car) and Cyt b_{559} demonstrated the same rate of electron transfer to $\text{P}_{680}^{\bullet+}$, shown by similar relative yields of their EPR spectra compared to the S_2 state spectrum at cryogenic temperature, indicating that Cyt b_{559} donates an electron to $\text{P}_{680}^{\bullet+}$ through an intermediate, redox-active Chl or Car, and that the slower step is donation to $\text{P}_{680}^{\bullet+}$ [33].

However, it has been suggested [21] that the increasing oxidation of the secondary donors compared to S_2 state formation as the temperature is decreased is not a result of competing rates, but instead a result of freezing PSII in conformations in which Y_Z cannot be oxidized and thus the S_2 state cannot be formed. In this population of centers, the oxidation of secondary donors competes with the slower rate of $\text{P}_{680}^{\bullet+}\text{Q}_A^{\bullet-}$ charge recombination ($t_{1/2} \sim 1\text{--}5\ \text{ms}$ at cryogenic temperature), and not Y_Z donation ($t_{1/2} = 4.2\ \mu\text{s}$) [8,21].

Further characterization of $\text{Car}^{\bullet+}$ and $\text{Chl}^{\bullet+}$ was subsequently obtained from spectroscopy on a variety of sample types. PSII-enriched membranes from higher plants contain all of the subunits of PSII, as well as peripheral LHC-II light harvesting complexes. PSII isolated from *Synechocystis* contains all of the PSII subunits present in that species, without any peripheral light harvesting complexes. Samples of PSII reaction centers contain only the D1/D2/Cyt b_{559} subunits and a limited number of pigments. PSII reaction center preparations can carry out the primary charge separation between P_{680} and Pheo; however, these preparations are unable to photooxidize Y_Z or Y_D , do not contain Q_A or Q_B , and have different portions of the protein exposed to solvent, potentially perturbing the redox potentials of the cofactors.

In PSII reaction centers, absorption spectroscopy showed the oxidation of Car [50], in addition to Chl [51], with bleaching of Car occurring more rapidly, and modification of neutral pigments was shown by HPLC [50]. The Car bleaching was also biphasic, implying that two Car were oxidized, one more rapidly than the other [50]. $\text{Chl}^{\bullet+}$ and $\text{Car}^{\bullet+}$ were also detected by FTIR spectroscopy on PSII-enriched membranes [52], and by ENDOR spectroscopy on both PSII-enriched membranes and PSII reaction centers [53]. Fluorescence spectroscopy on PSII-enriched membranes indicated that $\text{Chl}^{\bullet+}$ is a strong quencher of fluorescence with implications for photoprotection under high light [54]. Resonance Raman spectroscopy was performed on PSII reaction centers [55] and on PSII isolated from *Synechocystis* [56,57], where the observation that Cyt b_{559} must be pre-oxidized to observe $\text{Chl}^{\bullet+}$ and $\text{Car}^{\bullet+}$ indicated that these cofactors are members of the same electron-transfer pathway [57]. Electron transfer along this pathway was observed experimentally in PSII-enriched membranes solubilized with 0.5% β -DM, where $\text{Car}^{\bullet+}$ formed by illumination at liquid helium temperatures was converted into $\text{Chl}^{\bullet+}$ upon warming [58], although the electron transfer may have been modified by the presence of detergent [20,59].

Unfortunately, $\text{Chl}^{\bullet+}$ and $\text{Car}^{\bullet+}$ sometimes have similar spectral signatures, including their 9 GHz (X-band) EPR spectra [57,60,61], and there has historically been some confusion regarding the conditions that result in the formation of $\text{Chl}^{\bullet+}$, $\text{Car}^{\bullet+}$, or both (see introduction in ref. [58]). Generally, it is true that illumination at low temperatures with preoxidized Cyt b_{559} generates both the $\text{Car}^{\bullet+}\text{Q}_A^{\bullet-}$ and $\text{Chl}^{\bullet+}\text{Q}_A^{\bullet-}$ charge separations from at least 6 to 190 K [20,60], and that $\text{Chl}^{\bullet+}\text{Q}_A^{\bullet-}$ is more stable than $\text{Car}^{\bullet+}\text{Q}_A^{\bullet-}$ as the temperature increases. Currently, $\text{Chl}^{\bullet+}$ and $\text{Car}^{\bullet+}$ can be distinguished by several forms of spectroscopy, including high-field EPR [60,61] and near-IR absorption (see Section 5), among others.

3. Properties of Cyt b_{559}

Cyt b_{559} is composed of one α - and one β -subunit, each providing one His ligand to the heme (shown in Fig. 1B), and is an integral part of PSII adjacent to the D2 subunit [62]. Interestingly, Cyt b_{559} displays a range of redox potentials in PSII, from -150 to $+390\ \text{mV}$ [63,64], as determined by redox titrations. The redox potential has been observed to shift following different treatments to PSII, and it has been hypothesized that the shift in redox potential may play a functional role in decreasing photoinhibition *in vivo* [41]. Both the photooxidation and photoreduction (at higher light levels) of Cyt b_{559} have been observed [36,65]. The structure, spectroscopic properties, and electron-transfer reactions of Cyt b_{559} have been previously reviewed [19,25–27].

3.1. LP/HP interconversion

A conversion from HP to LP or IP Cyt b_{559} has been observed under a variety of conditions, including the removal of lipids [66], Tris washing [67–69], the removal of Car [70], heat treatment [69,71–73], salt washing [74,75], the removal of the 17- and 23-kDa extrinsic subunits [49,63], alkaline pH incubation [69], and high light illumination at $5000\ \mu\text{E}/\text{m}^2\text{s}$ [76], perhaps due to the complete reduction of Q_A and Q_B [41]. However, the conversion from LP to HP Cyt b_{559} has proven to be more difficult to achieve experimentally, although it has been observed that the Cyt b_{559} present in the stromal membranes is mainly in the LP form, while the HP form is enriched in the appressed regions, indicating that a conversion process takes place *in vivo* [77]. Experimentally, a 50–60% restoration of HP Cyt b_{559} has been achieved by the addition of PQ to heptane-extracted chloroplasts [70]. The incorporation of PSII core complexes, which contain only LP Cyt b_{559} , into liposomes composed of digalactosyldiacylglycerol and phosphatidylcholine resulted in a conversion of a portion of the LP Cyt b_{559} into the HP form (as defined by reducibility by 20 mM hydroquinone), while a slightly greater portion remained in the LP form [78]. In Mn-depleted PSII samples, it was observed that reducing conditions and anaerobicity resulted in the conversion of 60% of Cyt b_{559} into the HP form, which was reversible upon the addition of oxygen, and the conversion was absent in O_2 -evolving PSII samples [79]. It has been hypothesized that binding of PQ at a site near Q_B may increase the redox potential of HP Cyt b_{559} [80,81]. However, despite extensive study, a method for reversibly converting 100% of Cyt b_{559} from the HP to LP form has not been discovered, and the structural and functional differences between the HP, IP, and LP forms have not been identified. It is likely that the differences in redox potential are due to the protein environment surrounding the heme, and not to changes in the ligation of the His residues, based on the similar EPR spectra observed for the different forms [63] and the structural similarity of the LP heme to model complexes [82]; however, it has also been observed that the HP form has a different absorbance signature in the Soret region of the visible spectrum [64]. Well-defined structural characterization of the different forms of Cyt b_{559} , and methods for their interconversion, will yield insight on the functional role of each form.

3.2. Site-directed mutagenesis on Cyt b_{559}

Although many mutations to Cyt b_{559} prevent the assembly of PSII, some site-directed mutants have been successfully isolated. Site-directed mutation of the heme-binding His23 ligand from the α subunit to Tyr or Met yielded PSII complexes that lacked the heme of Cyt b_{559} , either due to its total absence from the complex or to loss during isolation of PSII, and were as capable of oxygen evolution as WT, but more sensitive to photodamage [83]. The mutation in the β subunit of Phe26, about 15 Å away from the Fe approximately in the plane of the heme, to Ser resulted in PSII that retained a reduced PQ pool in the dark. In contrast, the PQ pool of WT PSII undergoes reoxidation in the dark [84,85], which suggests that Cyt b_{559} may be involved in the oxidation of

the PQ pool in the dark [86,87]. A mutation to the axial heme ligand in the α subunit, His22Lys [88], and mutation of a residue near the heme, Tyr18Ser, resulted in mainly LP Cyt b_{559} and increased photoinhibition compared to WT, attributed to a change in ligation of the heme and to a change in hydrophobicity near the heme, respectively [89]. The Arg7Leu mutation, nearby the heme, resulted in heme ligand displacement and enhanced photoinhibition [90]. LP Cyt b_{559} was also formed in cells with a deletion in the gene for the Psb30 protein, and photoinhibition was again increased [91]. Overall, the characterization of PSII containing mutations in Cyt b_{559} indicates that Cyt b_{559} plays a role in both the structure of PSII and in photoprotection of the protein.

4. Structure

4.1. Structure of each cofactor

Resonance Raman, high-field EPR, ESEEM, and HYSCORE spectroscopies have provided structural information about the individual cofactors involved in secondary electron transfer within PSII. The conformation of the cofactors affects their redox potentials as well as the edge-to-edge distance for electron transfer. Resonance Raman spectroscopy has shown that the redox-active carotenoids in *Synechocystis* PSII are in an all-*trans* configuration [57,92–94]. High-field EPR spectroscopy has been used to separately resolve the signals from $\text{Car}^{\bullet+}$ and $\text{Chl}^{\bullet+}$, indicating that the structure of $\text{Car}^{\bullet+}$ is similar to a canthaxanthin radical with a slightly rhombic tensor, while $\text{Chl}^{\bullet+}$ is similar to monomeric $\text{Chl}^{\bullet+}$ in methylene chloride [60,95]. Pulsed ^1H ENDOR spectroscopy has indicated that the $\text{Car}^{\bullet+}$ in PSII are as symmetrical as $\text{Car}^{\bullet+}$ *in vitro*, with the radical delocalized along the polyene chain [96]. This interpretation is also supported by high-field EPR [97], resonance Raman spectroscopy [98], and density functional theory (DFT) calculations [99].

By comparison to the selectively ^{15}N -labelled model complex Fe(III)–protoporphyrin IX (Im)₂, it was determined that the structure of the heme in LP Cyt b_{559} is very similar to that of the model complex, with the unpaired electron in a non-bonding iron orbital [82]. Overall, it seems that the cofactors in PSII are in similar conformations as the compounds dissolved in solution; therefore, any unusual redox properties are most likely due to their protein surroundings, including the unusually high and variable redox potential of Cyt b_{559} .

4.2. Location of each cofactor

4.2.1. Spectroscopic measurements

Higher resolution crystal structures have shown the location and arrangement of each cofactor (see Section 4.2.2). However, there has been some difficulty in localizing or distinguishing each component, most notably Car, which has an appearance similar to a phytol tail of Chl, Pheo, or PQ. In addition, crystallography cannot identify which Chl and Car molecules are redox active. Hence, other spectroscopic techniques have been used to determine the locations of specific redox cofactors with respect to others. The spin-lattice relaxation rates calculated from pulsed high-frequency EPR spectroscopy indicated that on average $\text{Car}^{\bullet+}$ is 38 ± 1 Å from the non-heme iron, and the average $\text{Chl}^{\bullet+}$ is ≥ 40 Å from the non-heme iron [100]. Consistent with those values, X-band saturation-recovery EPR experiments have shown that the weighted average of $\text{Car}^{\bullet+}$ and $\text{Chl}^{\bullet+}$ is 39 ± 2.5 Å from the non-heme iron [101]. Pulsed EPR spectroscopy has shown that the OEC, Q_A , and $\text{Chl}^{\bullet+}$ are 27 Å [102], 38.5 Å, and 29.4 Å from Y_D [103,104], respectively, while Y_2 and Y_D are both 37 ± 5 Å from the non-heme iron [105].

Relative orientations of the redox-active cofactors have also been determined. Pulsed EPR spectroscopy experiments have yielded the angular relationship between Y_2 , Y_D , and the OEC [106], and the vector between Y_D and $\text{Chl}^{\bullet+}$ and the membrane normal has been found to be $50 \pm 5^\circ$ based on a distance of 29.5 Å between Y_2 and Y_D [107,108]. By HYSCORE spectroscopy, it has been shown that one indole nitrogen

from a tryptophan residue interacts with $\text{Car}^{\bullet+}$ [109]. $\text{Chl}^{\bullet+}$ species, generated by illumination at 198 K, on average have ring planes oriented perpendicular to the membrane plane based on high-frequency EPR [61], and two spectrally-separable Car species have been observed by linear dichroism of PSII core complexes with one oriented parallel to the membrane plane and one tilted approximately 45° [110,111].

Based on computational structural analysis [112], rates of energy transfer observed by transient absorption difference spectroscopy [113], absorbance spectroscopy [114], and electrochromic difference spectroscopy [115], it was proposed that D1-His118 and D2-His117 were the ligands to the accessory Chls closest to P_{680} . This was supported by disrupted exciton transfer in site-directed mutants of D2-His117 and D1-His118 [116–118] and by the characteristic growth of the mutants [119,120]. Based on saturation-recovery EPR spectroscopy and homology to the bacterial reaction center, it was further proposed that D1-His118 and D2-His117 were ligands to the redox-active Chls named “ $\text{Chl}_Z^{\bullet+}$ ” [101]. This hypothesis was confirmed by perturbed resonance Raman spectra of a Mn-depleted D1-H118Q *Synechocystis* PSII sample [121] following selective excitation into the 820 nm absorbance band of $\text{Chl}^{\bullet+}$ [56]. Interestingly, the EPR and near-IR spectra of the mutant were similar to those of wild type [121]. $\text{Chl}^{\bullet+}$ has been observed to quench fluorescence [54], and mutational studies of D1-His118 ligated to Chl_{ZD1} (historically known as Chl_Z) and D2-His117 ligated to Chl_{ZD2} (historically known as Chl_D) indicated that mutations around Chl_{ZD2} decreased the amount of fluorescence quenching in cyanobacteria [122,123]. In contrast, FTIR spectroscopy has indicated that both Chl_{ZD1} and Chl_{ZD2} may be photooxidized in higher plants [124]. The location of $\text{Chl}^{\bullet+}$ remains uncertain, and may have variability between species.

4.2.2. X-ray crystallography

Low-resolution cryo-electron microscopy has shown the general structure and trans-membrane helix positions in PSII [125–130], while recent X-ray crystallography [40,131–140] studies have provided a clear picture of the overall structure of PSII and the locations of all of the cofactors; however these structural methods cannot identify which of the Chl and Car cofactors are redox-active and involved in secondary electron transfer. As shown in Figs. 1A and 2, Car_{D2} is the closest secondary donor to P_{680} , and it is likely that this Car is the initial secondary donor, which is also consistent with spectroscopic studies [58]. Shown in Fig. 2, once Car_{D2} has been oxidized, there are many adjacent cofactors that may be involved in secondary electron transfer. These include Chl_{ZD2} , Cyt b_{559} , and other Car and Chl cofactors. Hence, it is possible that an oxidizing equivalent on Car_{D2} could be transferred to a wide selection of the closely spaced 35 Chl and 11 Car molecules per PSII when Cyt b_{559} is preoxidized.

Carotenoids are found in the periphery of PSII cores, spanning the membrane, with many subunits involved in Car binding. Chlorophylls are clustered together primarily in CP43 and CP47, with some in close proximity to Car [141]. Distances between cofactors, indicated in Fig. 2, include 11.1 Å from B_B to Car_{D2} and 11.6 Å from Car_{D2} to Cyt b_{559} . These short distances provide an efficient pathway for electron transfer from P_{680} to Cyt b_{559} via Car_{D2} . From Car_{D2} , Chl_{ZD2} is 4.9 Å away, which is in turn 14.4 Å away from a Chl molecule in close proximity to the other Chl/Car cofactors in CP47. Car_{D2} is also 10.7 Å away from another Car in close proximity to the other Chl/Car cofactors in CP43, one of which is 15.4 Å from Chl_{ZD1} . Within CP43 and CP47, the distances between Chl/Car cofactors range from less than 4 Å to around 8 Å, with almost all distances less than 6 Å. The redox potentials of $\text{Car}^{\bullet+}$ and $\text{Chl}^{\bullet+}$ are relatively similar [142] and the Chl/Car cofactors are arranged with short distances for single electron-transfer steps; therefore, the radical hole may quickly equilibrate throughout the Chl/Car cofactors, possibly into CP43 and CP47, as shown in Fig. 2.

From the 1.9 Å resolution crystal structure [40], the redox-active Car in D2 is approximately parallel to the membrane plane, 11 Å from Cyt b_{559} , 5.0 Å from Chl_{ZD2} , 11 Å from another Car, and passes within 10 Å of

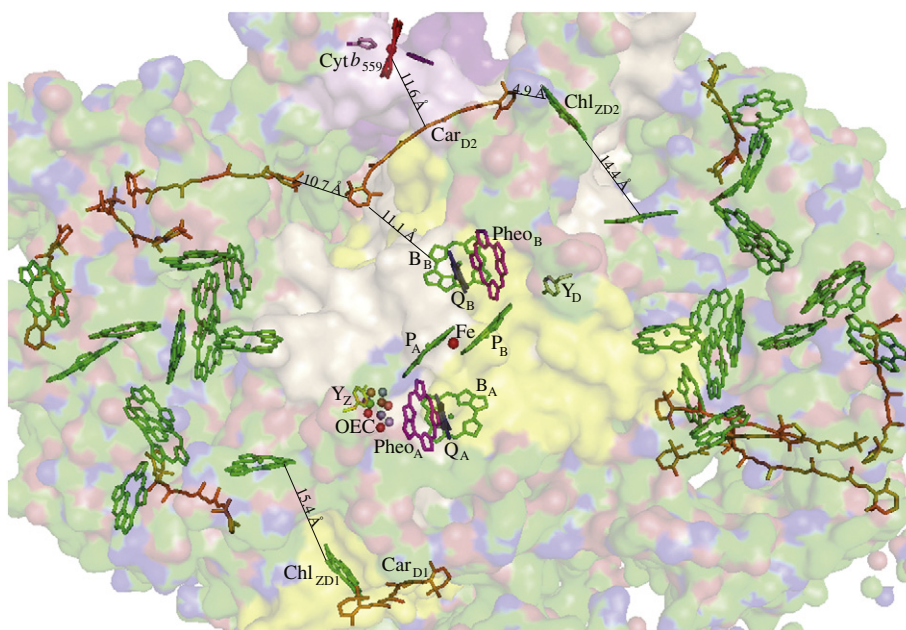


Fig. 2. The arrangement of cofactors in PSII, viewed from the membrane surface (PDB ID: 3ARC). The oxygen-evolving complex (OEC) is shown with manganese atoms in purple, oxygen in red, and calcium in green; tyrosine Z (Y_Z) is shown in yellow and tyrosine D (Y_D) is shown in tan; chlorophylls (Chl) are shown in green; β -carotene (Car) is shown in orange; pheophytins (Pheo_A and Pheo_B) are shown in magenta; and quinones (Q_A and Q_B) are shown in blue. The surfaces of the D1, D2, α - and β -Cyt b_{559} subunits are colored yellow, tan, pink and purple, respectively, with the remaining surfaces colored according to atom identity. The longest distances (in Å) between neighboring cofactors are shown as solid lines, with the unlabelled distances being mainly less than 6 Å.

the tails of Q_B and B_B. Car_{D2} is 28 Å from the non-heme iron, which is shorter than the average 38 Å observed by saturation recovery EPR [100], indicating that the other Car^{•+} (see Section 5) is further from the non-heme iron, perhaps in CP43 or CP47. Chl_{ZD2}, in addition to the proximity to Car_{D2}, is approximately 14 Å from a Chl in CP47, in which the cofactors are very closely spaced, and 6.0 Å from the tail of Q_B. Car_{D1} is approximately perpendicular to the membrane plane, 5 Å from Chl_{ZD1}, 13 Å from the tails of Q_A, Pheo, and B_A, and 35 Å from the non-heme iron. Chl_{ZD1} is approximately 16 Å from both Chl and Car in CP43. The cofactors in CP43 and CP47 are bridged by Chl_{ZD2}, 14 Å from CP47, through Car_{D2} and its adjoining Car 11 Å away, which is 3.9 Å from the cofactors grouped in CP43. Several negatively charged lipids are located near Chl in CP43 (4.3 Å away) and CP47 (5.3 Å away), which have been hypothesized to decrease the redox potential of Chl, resulting in more stable radicals [143,144]. Negatively charged lipids are also located 5.5 Å away from a Car in CP47, and 6.1 Å away from Car_{D1}. The observation by HYSCORE that Car^{•+} is coupled to the N of a Trp is supported by the fact that Car_{D2} is 7.8 Å from a Trp residue [109]. Additionally, Trp residues are close to several other Car molecules, including 5.1 Å from a Car in CP43.

The heme of LP Cyt b_{559} is ligated by one His from the β -subunit of Cyt b_{559} (*psbF* gene product) at a distance of 2.15 Å, with the imidazole ring bisecting the N-Fe(III)-N angle, and one His from the α -subunit (*psbE* gene product) at 2.03 Å with the imidazole ring at an angle of approximately 22.5° to the other. The plane of the heme is slightly twisted.

4.2.2.1. Q_C site. The 2.9 Å resolution crystal structure has provided some evidence that binding of PQ to PSII may occur at a site other than Q_B in the hydrophobic cavity near Cyt b_{559} [138,144]. Binding of inhibitors has previously been observed at a site separate from Q_B, which could modulate the environment of the heme in Cyt b_{559} , its redox potential, and its electron-transfer properties [80,87]. However, occupancy of the Q_C site was not observed in the 1.9 Å resolution crystal structure [40], possibly due to higher disorder in the binding of PQ to the site [138] stemming from low affinity or non-specific binding, or to loss during preparation of the crystals.

4.2.2.2. Calculations from X-ray structures. A wide variety of spectroscopic properties may be calculated using distances from the X-ray structures in conjunction with quantum parameters. Possible energy transfer pathways [145] and the ability of Chl^{•+} to quench fluorescence [146] have been modeled and compared with experimental data. The redox potentials of Chl and Car have also been calculated based on crystal structures up to 3.0-Å resolution, and it has been found that the redox potential of Car and Chl in D1 are higher than those in D2, which may indicate a preference for radical localization in D2 [142,147], although these potentials may be modified by slight conformational changes in the protein. The calculation of CD and LD spectra, excitonic coupling, domain structure, site energies, and exciton transfer from structural data has been previously reviewed in Ref. [141].

5. Progress in characterization of secondary electron transfer

Following the identification of the redox-active secondary cofactors (see Section 2), it was shown that Cyt b_{559} , Car^{•+}, and Chl^{•+} are members of the same secondary electron-transfer pathway. Most of the studies of the secondary electron-transfer reactions have been carried out by illuminating PSII at cryogenic temperatures, under which conditions PSII is limited to a single stable charge separation [30]. When Cyt b_{559} is prereduced, it is the preferential (lowest potential) electron donor at cryogenic temperatures. Its quantitative photooxidation upon illumination, to generate the Cyt b_{559}^{ox} Q_A^{•-} charge separation, can be observed by EPR [30] and optical spectroscopy [8]. However, low-temperature illumination of a PSII sample in which Cyt b_{559} is preoxidized generates a distribution of Chl^{•+} Q_A^{•-} and Car^{•+} Q_A^{•-} charge-separated states. In contrast, Y_D oxidation is on a separate pathway, which increasingly competes with the secondary electron-transfer pathway at higher pH [148].

Although the side-path donors do not operate at a high efficiency, organisms with enhanced cyclic electron transfer have been observed to be more resistant to photodamage [149,150]. Additionally, at 77 K, the Cyt b_{559}^{ox} /Q_A^{•-} charge separation can be generated in almost 100% of PSII centers with pre-reduced Cyt b_{559} [8]. At room temperature, the yield of oxidized Cyt b_{559} in PSII-enriched membranes inactive for O₂ evolution

following a single flash was approximately double (4.7%) the yield in an O_2 -evolving sample, and the maximum yield of oxidized Cyt b_{559} was reached after 21 s of continuous illumination in an O_2 -evolving sample, compared to 1.8 s for an inactive sample [36]. Due to the highly-oxidizing nature of P_{680}^{++} , the secondary donors, including the initial donor Car_{D2} , must be partially spatially separated from P_{680} so that their oxidation does not compete with water oxidation at the OEC under physiological conditions [24]. However, they may play a photoprotective role in low yield when the lifetime of P_{680}^{++} is long.

Species differences in the secondary electron-transfer pathway have been noticed between higher plants and cyanobacteria. For example, at high pH, Y_D^{\bullet} was formed in 75% of PSII centers in *Synechocystis* and 55% of centers in spinach [148]. The rate of P_{680}^{++}/Q_A^- recombination is slower in cyanobacterial PSII than in higher plant PSII [1,5]. The yield and identity of Chl^{*+} have also been observed to vary between plant and cyanobacterial PSII [20]. These differences may reflect differential mechanisms of photoprotection, such as the addition of the xanthophyll cycle in higher plants, or changes in auxiliary proteins since the divergence of photosynthetic eukaryotes and cyanobacteria [151].

Although electron transfer is known to occur among Cyt b_{559} , Car^{*+} , and Chl^{*+} , the sequence of events and electron-transfer partners are not well defined. The rate of electron transfer depends on the distance between cofactors, the difference in redox potentials, and on the reorganization energy required [152]. The distribution of Chl^{*+} , Q_A^{*-} and Car^{*+} charge-separated states generated at low temperature has been interpreted to arise from the trapping of a distribution of conformations of PSII that have slightly different redox potentials of the Chl and Car cofactors when a sample of PSII is frozen [20]. Because Chl and Car generally have similar redox potentials, for a given conformation of PSII, either one of the Chl or one of the Car species will have the lowest redox potential and become the final electron donor when an oxidizing equivalent equilibrates among the secondary Chl/Car electron donors. Using the 3.7 Å resolution crystal structure [132], an electron-transfer pathway from P_{680} to Car_{D2} to Chl_{ZD1} was proposed involving a series of Chls in CP43 [146]. Although many electron transfer steps are required to reach Chl_{ZD1} , an electron will tunnel to any redox center with the correct midpoint potential, whenever the distance through other cofactors is short enough, even if an individual step is unfavorable [152].

Different PSII preparations, containing a variable number of subunits, may result in different solvent exposure and hence redox potentials for the cofactors. A PSII reaction center complex, containing only D1/D2/Cyt b_{559} , has the highest solvent exposure, whereas PSII core complexes, lacking the LHC-II proteins, have an intermediate amount of solvent exposure compared to PSII-enriched membranes or chloroplasts. These differences may explain, in part, differences in the secondary electron-transfer reactions in these different types of PSII preparations.

Near-IR spectroscopy has been a valuable tool for elucidating the relationships between the secondary donors under a variety of conditions. The near-IR spectrum of PSII (Fig. 3) exhibits the strong $D_0 \rightarrow D_2$ transition of Car^{*+} near 990 nm with an extinction coefficient of $160,000 \text{ M}^{-1} \text{ cm}^{-1}$ [153]. Chl^{*+} has a weaker absorbance at 800 to 850 nm with an extinction coefficient of $7000 \text{ M}^{-1} \text{ cm}^{-1}$ [154]. In addition, an absorbance at 750 nm is attributed to a neutral β -carotene radical (Car^{\bullet}) [155]. Recently, an absorbance at 1450 nm attributed to the $D_0 \rightarrow D_1$ transition of Car^{*+} has also been observed [156].

5.1. Properties of secondary electron transfer in PSII with mostly LP/IP Cyt b_{559}

5.1.1. Higher plant PSII containing LP/IP Cyt b_{559}

In active, O_2 -evolving samples, Cyt b_{559} is mostly in the HP and IP form [63,64], and predominantly reduced under ambient conditions, while inactive, non- O_2 -evolving samples contain the IP and LP forms, which are typically oxidized under ambient conditions. In a study of inactive PSII reaction center complexes (D1/D2/Cyt b_{559}) from spinach,

it was observed by resonance Raman spectroscopy that two Car molecules were photooxidized, one more easily than the other, and at least three Chl molecules were also photooxidized [93]. These results supported work done on inactive PSII reaction center complexes (D1/D2/Cyt b_{559}) from pea plants [157]. By near-IR spectroscopy on PSII-enriched membranes, two Chl^{*+} bands were observed, attributed to Chl_{ZD1}^{*+} (" Chl_Z^{*+} ") and Chl_{ZD2}^{*+} (" Chl_D^{*+} ") and it was observed that Car^{*+} decayed mainly by recombination with Q_A^{*-} , but also partly by the formation of Chl^{*+} absorbing at 850 nm [20].

5.1.2. Cyanobacterial PSII containing LP/IP Cyt b_{559}

In non- O_2 evolving, inactive *Synechocystis* PSII samples, one Chl^{*+} peak was observed by near-IR spectroscopy, in contrast to two Chl^{*+} peaks observed in inactive spinach PSII samples, and it was attributed to Chl_{ZD1}^{*+} (" Chl_Z^{*+} ") [20,143]. The Car^{*+} peak in inactive *Synechocystis* PSII samples was observed to shift as the radical decayed, indicating the presence of two redox-active Car absorbing at 982 and 1027 nm that were affected differently by the presence of Y_D^{\bullet} in the sample [143,158]. Car^{*+} in these samples decayed only by recombination with Q_A^{*-} and not by conversion to Chl^{*+} , and Car^{*+} decayed more rapidly than Chl^{*+} by recombination with Q_A^{*-} , indicating its closer proximity to Q_A [20].

Illumination of PSII samples with a mixture of oxidized LP Cyt b_{559} and reduced HP Cyt b_{559} results in the formation of Car^{*+} and Chl^{*+} in the PSII centers with LP Cyt b_{559} , whereas Cyt b_{559} oxidation occurred in the remainder of the centers. In PSII samples with oxidized LP Cyt b_{559} , the peak from Car^{\bullet} , which may be involved in fluorescence quenching, was not observed at 750 nm, and the wavelength maximum of the Car^{*+} peak was shifted to the red compared to centers containing oxidized IP and HP Cyt b_{559} . These changes indicate that different Chl and Car cofactors are oxidized in centers containing different redox forms of Cyt b_{559} , implying different pathways and/or outcomes of secondary electron transfer in response to shifts in the midpoint potential of Cyt b_{559} [159].

5.2. Properties of secondary electron transfer in PSII with mostly HP Cyt b_{559}

5.2.1. Higher plant PSII containing HP/IP Cyt b_{559}

In samples of O_2 -evolving PSII-enriched membranes solubilized with 0.5% β -DM, near-IR spectroscopy showed that the Car^{*+} peak had a wavelength maximum around 1010 nm. Compared to non- O_2 -evolving, inactive spinach samples of PSII-enriched membranes, the $t_{1/2}$ for the formation of Car^{*+} absorbing at 970 nm was similar to that in the O_2 -evolving samples, but more Chl^{*+} absorbing at 820 nm remained after a series of flashes, implying an altered secondary electron transfer pathway between active and inactive spinach PSII centers [160].

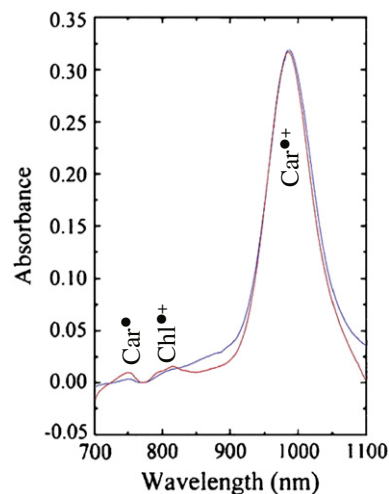


Fig. 3. Light-minus-dark difference spectra of Mn-depleted *Synechocystis* PSII (blue) and O_2 -evolving *Synechocystis* PSII (red); modified from Ref. [159].

5.2.2. Cyanobacterial PSII containing HP/IP Cyt b_{559}

In contrast to non- O_2 -evolving, inactive cyanobacterial samples, O_2 evolving samples contained Chl^{*+} peaks with more structure and a narrower Car^{*+} peak, perhaps due to a more homogeneous protein structure in the presence of the OEC [143]. The Car^{*+} peak again contained contributions from two different Car^{*+} cofactors absorbing maximally at 984 and 1029 nm; however, the Chl^{*+} peaks contained contributions from five spectrally distinct Chl^{*+} cofactors, rather than one or two, which formed and decayed at different rates. The amount of some Chl^{*+} species continued to increase after total charge separation had been achieved, indicating conversion among the different Chl^{*+} , and also perhaps between the Car^{*+} , with the most stable Chl^{*+} absorbing at 810 nm [143].

5.3. Properties of secondary electron transfer in PSII with modified pigments

A portion of the pigments in PSII complexes may be removed by washing with Triton-X 100 [161] or using Cu-immobilized chromatography [162]. It has been observed in these systems that Car is capable of quenching 1O_2 [161], and that secondary electron transfer is possible in the D1 subunit [162] at room temperature [163]. Site-directed mutagenesis of D1-Thr179, nearby to Chl B_A , indicated that the presence of Car_{D2}^{*+} , Chl_{D2}^{*+} , and Q_A^- resulted in a blue shift of the Chl B_A Q_Y absorbance band [164].

PSII will not assemble without Chl [119,120] or Car [165], which indicates a structural requirement for the cofactors. However, genetic manipulation of the carotenoid biosynthesis genes in *Synechocystis* resulted in the growth of cells containing β -zeacarotene, with 9 conjugated double bonds and only one β -ionylidene ring, instead of β -carotene, which contains 11 conjugated double bonds and two β -ionylidene rings [165]. PSII isolated from these cells displayed a Car^{*+} absorbance maximum at 898 nm and a higher ratio of Chl^{*+}/Car^{*+} , both consistent with the shorter conjugation length and higher redox potential of β -zeacarotene.

6. Summary

The photooxidation of the secondary electron donors, Cyt b_{559} , Car, and Chl, has been observed in PSII under a variety of conditions. Although the yield of photooxidized secondary donors is low under physiological conditions, they are believed to play a photoprotective role when electron transfer resulting in water-oxidation catalysis is inhibited. Cyt b_{559} , with LP, IP, and HP forms, is preferentially oxidized by P_{680}^{*+} through a network of Car and Chl cofactors. Cyt b_{559} may also be reduced by the acceptor side of PSII, thereby forming a cyclic electron-transfer pathway that connects the donor and acceptor sides of PSII to remove potentially harmful, excess oxidizing equivalents. The structure of Cyt b_{559} has been characterized by X-ray crystallography, but the structural differences between the LP, IP and HP forms of Cyt b_{559} remain to be determined, as does the physiological role for their conversion. The structure and arrangement of Car and Chl cofactors have also been determined by X-ray crystallography, and their redox activity has been probed by a variety of spectroscopic methods. Electron transfer among the secondary donors involves many Car and Chl cofactors, especially in O_2 -evolving samples, which appear to be connected in a network extending into the peripheral subunits of PSII (CP43 and CP47), rather than involving only a few cofactors in a simple pathway. The sequence of electron-transfer steps and the location of each redox-active Car and Chl cofactor, however, remain to be determined.

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